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Mechanism of Escape of Endogenous Murine Leukemia Virus *emv-14* from Recognition by Anti-AKR/Gross Virus Cytolytic T Lymphocytes

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It was previously shown that spleen cells from endogenous ecotropic murine leukemia virus *emv-14*$^+$ AKXL-5 mice fail to stimulate an anti-AKR/Gross virus cytolytic T-lymphocyte (CTL) response in a mixed lymphocyte culture with primed C57BL/6 responder spleen cells, whereas spleen cells from AKXL strains carrying the very similar *emv-11* provirus do stimulate a response (Green and Graziano, Immunogenetics 23:106–110, 1986). We wished to determine whether the lack of response with AKXL-5 spleen cells was at the level of recognition between effector cell and target cell and whether the relevant mutation was within the *emv-14* provirus. It is shown here that EMV-negative SC-1 fibroblast cells transfected with the major histocompatibility complex class I K^b gene and infected with virus isolated from the AKXL-5 strain (SC.K^b/5 cells) were not lysed by *H-2*^{b}-restricted anti-AKR/Gross virus CTL. SC.K^b cells infected with virus isolated from *emv-11*$^+$ strains, however, were efficiently lysed by anti-AKR/Gross virus CTL, indicating that there is nothing intrinsic to EMV-infected SC.K^b cells that would prevent them from being recognized and lysed efficiently by anti-AKR/Gross virus CTL. Analysis of virus expression for the infected SC.K^b cells by XC plaque assay and by flow cytometry indicated that *emv-14* virus expression for SC.K^b/5 cells was not significantly different from that for *emv-11*-containing SC.K^b/9 or SC.K^b/21 cells. These data show that the mutation responsible for the lack of CTL recognition and lysis is at the level of recognition between target cell and effector cell. Furthermore, these data strongly suggest that the mutation is within the *emv-14* genome. Flow cytometry experiments with monoclonal antibodies against a number of viral determinants indicated that there was no gross mutation detectable in the viral determinants analyzed. The data suggest that the relevant mutation may be a point mutation or a small insertion or deletion within a coding sequence that is critical for CTL recognition.

Endogenous ecotropic murine leukemia viruses (EMVs) are present in the highly leukemia-susceptible AKR strain of mouse and are passed on to succeeding generations through the germ line. EMVs recombine with other endogenous murine leukemia virus sequences to form the mink cell focus-inducing (MCF) virus, which is thought to be directly responsible for the high incidence of leukemia in AKR mice (27, 31). The importance of EMV in the disease process is underscored by the correlation of high EMV titers in newborn mice with an increased incidence of leukemia (37). In addition, EMV proviruses have been associated with malignant lymphoma (47).

EMV proviruses are distinguished from each other by their characteristic integration sites in the AKR genome. The sizes of the different AKR EMV integrants are conserved, as shown by restriction endonuclease site mapping with *Pst*I, which cleaves within the long terminal repeats (LTRs), giving an 8.2-kilobase (kb) fragment (8, 59). Certain internal restriction sites within the different integrants are also conserved (29). The endogenous EMV proviruses found in AKR/J mice (*emv-11*, *emv-13*, and *emv-14*) independently segregate from each other in the AKXL (AKR/J and C57L progenitor strains) recombinant inbred strains of mice (59). The use of these AKXL strains allowed Green and Graziano (21) to determine that, of these three proviruses, only *emv-11* was capable of stimulating an anti-AKR/Gross virus cytolytic T-lymphocyte (CTL) response. Irradiated splenic stimulator cells from *emv-11*-positive AKXL mice served as a source of viral antigen in secondary in vitro mixed lymphocyte cultures to induce the generation of spleen cells from tumor-primed C57BL/6 responder mice. Spleen cells from AKXL mice that inherited either the *emv-13* or *emv-14* provirus were unable to stimulate an anti-AKR/Gross virus response, however. The failure of *emv-13*$^+$ spleen cells to stimulate a response is expected, since *emv-13* is known to code for a defective virus (9), and mice that have inherited only *emv-13* are not viremic (59). The failure of *emv-14* to stimulate a response occurs despite the fact that *emv-11*$^-$ *emv-14*$^+$ AKXL-5 mice develop viremia (59; confirmed and quantitated here), indicating that the virus must be replication competent, transcriptionally functional, and able to form budding particles with functional envelope antigens which allow the infection to spread laterally. This failure to stimulate a response is surprising in light of the notion that *emv-14* was derived by reintegration of an infectious primordial *emv-11* provirus (59).

One possible explanation for the failure of *emv-14*$^+$ spleen cells to stimulate anti-AKR/Gross virus CTL is that there is a coding sequence mutation within the viral genome at a site critical for CTL recognition, within the gp70 envelope gene, for example. A coding sequence mutation could allow *emv-14*-positive tumor cells to avoid lysis by, and prevent clonal expansion of, antiviral CTL. This would represent one mechanism by which tumors may escape cellular immunity and become established. There is suggestive evidence that the gp70 envelope antigen is a major determinant for anti-AKR/Gross virus CTL (16, 20, 40). Other loci within the murine genome, such as the *Fv-1* locus (18, 43) and the *H-2*

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locus (19, 35–37, 43), can also influence the development of antiviral immunity, however. In addition, the site of integration of provirus within the murine genome may influence CTL activity against viral antigens, by modulating the expression of viral antigens, for example. Prior to the present study, it was unknown whether the mutation responsible for the failure of *emv-14*$^+$ cells to stimulate antiviral CTL was within the proviral genome. It was also unknown whether the failure of *emv-14*$^+$ spleen cells to stimulate anti-AKR/Gross virus CTL was due to a defect at the level of recognition between the target cell and effector cell rather than at some prior step in the CTL stimulation process such as at the level of T-helper function.

In this study we show that when virus is isolated from *emv-11*$^+$ *emv-14*$^-$ AKXL-21 mice and used to infect the virus-negative SC-1 fibroblast cell line transfected with the *H-2K*^{b} gene (SC.K^b cells), the infected SC.K^b/21 cells are efficient targets for *H-2*^{b}-restricted anti-AKR/Gross virus CTL. Yet SC.K^b cells infected with virus from *emv-11*$^-$ *emv-14*$^+$ AKXL-5 mice (SC.K^b/5) cannot be lysed by anti-AKR/Gross virus CTL. These results indicate that the mutation responsible for the lack of CTL recognition and lysis is at the level of recognition between target cell and effector cell and furthermore that the mutation is almost certainly within the *emv-14* genome.

MATERIALS AND METHODS

Cells and strains. The SC-1 cell line is a continuous cell line from a wild mouse embryo (26). XC cells are a continuous line from a Wistar rat tumor induced with the Prague strain of Rous sarcoma virus and were originally obtained from Jan Svoboda. SC-1 and XC cells were cultured in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 30 μg of penicillin per ml, and 20 μg of streptomycin per ml. B.GV (53) and E♂G2 (22) are *H-2*^{b} Gross virus cell surface antigen (GCSA)-positive tumor cell lines derived from a BALB.B congenic mouse tumor and a C57BL/6 tumor, respectively, both induced with AKR/Gross virus passage A. AKR.H-2^b SL1 (SL1) and AKR.H-2^b SL1 clone 18-5 (cl.18-5) are GCSA$^+$ tumor cell lines derived from a spontaneous leukemia from an AKR.H-2^b congenic mouse (16, 22). AKR SL8 (SL8) is a GCSA$^+$ *H-2*^{k} tumor cell line derived from a spontaneous leukemia from an AKR/J mouse (15). YAC-1 (YAC) is an *H-2*^{a} tumor cell line that is used here as a highly natural killer cell (NK)-sensitive target cell and as an *H-2* haplotype-specific control for anti-*H-2*^{b} allogeneic CTL. All tumor cell lines (B.GV, E♂G2, SL1, cl.18-5, SL8, and YAC) were grown in RPMI supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 30 μg of penicillin per ml, and 20 μg of streptomycin per ml. All cells were maintained at 37°C in a 5% CO_2 atmosphere.

AKXL strains of mice, described by Steffen et al. (59), were obtained from Jackson Laboratories and have the following endogenous EMV genotypes: AKXL-5, *emv-14*; AKXL-9, *emv-11,13*; AKXL-16, *emv-11,13*; AKXL-21, *emv-11*; AKXL-28, *emv-13*; AKXL-29, *emv-11*$^-$,*13*$^-$,*14*$^-$. The EMV nomenclature has been used throughout this paper, replacing the older AKV nomenclature as follows: *emv-11* (*akv-1*), *emv-13* (*akv-3*), and *emv-14* (*akv-4*) (29).

RNA blot analysis. Total cellular RNA was isolated by the guanidinium-cesium chloride method (39), and 10 to 30 μg was blotted onto a nitrocellulose membrane by the manufacturer's recommended method (Schleicher & Schuell). The RNA blots were hybridized at 65°C overnight in a standard hybridization medium (4× SSCP [39], 1× Denhardt solution 0.1% [wt/vol] sodium dodecyl sulfate [SDS], 100 μg of denatured salmon sperm DNA per ml) (29). The *emv* probe used was derived from the gp70 region, a 334-base-pair (bp) *Sma*I fragment, from the AKR 623 *env* gene cloned into the *Eco*RI site of pBR322 (8, 28). This plasmid was constructed by D. R. Lowy and S. K. Chattopadhyay and was generously provided through B. A. Taylor. Only ecotropic DNA sequences and not xenotropic, amphotropic, or MCF murine leukemia viruses hybridize with this probe (8). We excised from this plasmid a 1,115-bp *Pst*I-*Hin*dIII fragment, containing the gp70 sequence, for radiolabeling by the random primer extension method (10). After hybridizations, the membranes were washed at 65°C for 30 min in each of the following conditions: 1× SSCP–0.1% SDS, 0.5× SSCP–0.1% SDS, and 0.1× SSCP–0.1% SDS. The washed filters were then exposed to X-ray film for autoradiography.

Virus isolation and quantitation by the XC plaque assay. Viruses were obtained from mouse tails in an alundum suspension, and the supernatant fractions were saved as viral extracts at −70°C (R. Bedigian, Jackson Laboratories, personal communication). Viral extracts from various AKXL strains were used to infect plates of SC.K^b or SC.D^b cells. AKR 623 ("*emv-11*") virus was kindly provided by Dr. Chattopadhyay, National Institutes of Health (38).

XC plaque assays (55; R. Bedigian, personal communication) were performed to quantitate the titers of infectious ecotropic virus from tail extracts or cell supernatant fractions. Briefly, 0.32% (wt/vol) Polybrene-treated SC-1 cells (2×10^5) were infected with virus for 5 days. On day 6, the infected cell sheet was irradiated with 254-nm UV light to stop cell growth. The infected monolayer was then overlaid with 1.2×10^6 XC cells. After syncytia were allowed to form for 3 days, the cell sheets were fixed with methanol and stained with 2% (wt/vol) methylene blue. Plaques were counted, and viral titers were determined.

Transfections. Major histocompatibility complex (MHC) class I genes were transfected into SC-1 cells by a modified version of the calcium phosphate-DNA coprecipitation protocol with 35 μg of plasmid DNA and the glycerol shock method (13, 14). The plasmids used for the transfections were clone I225 (3), carrying the *H-2K*^{b} and Neo^r genes, and clone I250 (69), carrying the *H-2D*^{b} gene with Moloney murine leukemia virus LTR promoter and the Neo^r gene. Both clones were kindly provided by Haemisch Allen, Gerald Waneck, and Richard Flavell at Biogen. Two days after transfection, 0.5 mg of G418 (GIBCO) per ml was added to the cells. One week later this medium was replaced with medium containing 1 mg of G418 per ml, and the cells were maintained at this concentration of drug for approximately 2 weeks and then maintained at 0.5 mg of G418 per ml.

Antibodies. Three monoclonal antibodies specific for certain MHC class I genes were used. HB41 secretes a monoclonal antibody, 28-13-3S, that is reactive with *H-2K*^{b}, and HB19 secretes a monoclonal antibody, 28-11-5S, that is reactive with *H-2D*^{b} (46). MCA169 is also reactive with *H-2D*^{b} (24, 32, 33). M1/42 is a monotypic monoclonal antibody with broad specificity for *H-2* class I antigens except that it does not react with *D*^{b} (57, 58).

Goat anti-Rauscher virus gp70 antiserum, with broad specificity for AKR/Gross, Friend, Moloney, and Rauscher murine leukemia virus group-specific gp70 antigenic determinants, was used for some of the viral antigen analyses. In addition, seven monoclonal antibodies specific for epitopes of gp70 were used. Four are ascites-derived mouse mono-

clonal antibodies: 16D7, 16C1, 19A2, and 16E4, corresponding to epitopes a, b, c, and d, respectively (60). Three are hybridoma supernatant fractions of rat origin: 35/56, 35/299, and 42/94, which correspond to epitopes f, g, and h, respectively (50). The 43-13 (AKR-specific anti-p12^{gag} antigen) and 43-17 (against a glycosylated *gag* polyprotein associated with *emv*) rat monoclonal antibodies were from concentrated hybridoma supernatant fractions (49).

Flow cytometric analysis. Cell surface antigen expression was measured by indirect immunofluorescence. Cells (2.5×10^5) were washed with staining medium (HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]-buffered RPMI 1640 [GIBCO] containing 0.2% [wt/vol] bovine serum albumin [fraction V; Sigma Chemical Co.]) and incubated with the appropriately diluted antibody for 30 min at 4°C. The samples were then washed twice in staining medium. Afterwards, the appropriately diluted fluorescein isothiocyanate-conjugated secondary antibody (goat anti-mouse, goat anti-rat, or swine anti-goat immunoglobulin) was added. Normal goat preimmune serum was used as a negative control for the goat anti-Rauscher gp70 antiserum. Background fluorescence values were determined with the following irrelevant monoclonal antibodies used as isotype controls: OKT8 (CRL 8014, mouse immunoglobulin G2a [IgG2a]) for MCA169, 16D7, 16C1, or 16E4; HB70 (mouse IgG3) for 19A2; 35.12 (mouse IgM, kindly provided by R. J. Noelle at Dartmouth Medical School) for HB41 or HB19; MAC-2 (TIB 166, rat IgG2a) for 35/56, 35/299, or 42/94; or MAC-1 (TIB 128, rat IgG2b) for 43/13 or 43/17. The cells were incubated at 4°C for 30 min and then washed once with staining medium. The samples were suspended in staining medium for flow cytometry analysis on an Ortho 50H cytofluorograph.

Cell sorting. SC-1-derived cell populations transfected with the K^b gene were sorted on an Ortho 50H cytofluorograph to obtain a subpopulation of cells expressing high concentrations of cell surface K^b antigen. The cells were then maintained in G418-free medium. Similarly, SC-1 cells transfected with the D^b gene were sorted to obtain a subpopulation of cells expressing high concentrations of D^b antigen.

Cytotoxicity assays. For the generation of allogeneic CTL directed against *H-2*^{b} target cells, responder DBA/2J (*H-2*^{d}) spleen cells were mixed with irradiated (2,000 rads) C57BL/6J (*H-2*^{b}) stimulator spleen cells at a responder-to-stimulator cell ratio of 2:1 on day 0. On day 5, fibroblast targets were labeled with ^{51}Cr overnight at 37°C. On day 6, tumor cells were labeled with ^{51}Cr as described by Green et al. (22), splenic effector cells were harvested, and the ^{51}Cr release assay was performed as described by Green (17) (see below).

For the generation of anti-AKR/Gross virus CTL, C57BL/6J mice were immunized with 2×10^6 B.GV cells. Responder spleen cells from immune mice were mixed with irradiated (8,000 rads) SL1 stimulator cells at a responder-to-stimulator cell ratio of 50:1. Effector cells were harvested and incubated with ^{51}Cr-labeled target cells at an effector-to-target cell ratio of 100:1, 20:1, and 4:1 at 37°C (6 h for fibroblast targets and 4 h for tumor targets).

RESULTS

RNA blot analysis of AKXL tissue. Since *emv-14*$^+$ AKXL-5 spleen cells failed to stimulate anti-AKR/Gross virus CTL with primed C57BL/6 responder cells (21), it was important to determine whether the *emv-14* provirus was transcriptionally active in AKXL-5 tissues relative to *emv-11*$^+$ AKXL-21 tissues. Total RNA was isolated from various AKXL liver or thymus tissues, blotted onto nitrocellulose filters, and hybridized with a DNA probe derived from the *env* gene and specific for EMV sequences (emv probe; see Materials and Methods). Figure 1 shows representative autoradiographic data. The amount of AKXL-5-derived RNA that hybridized to the *emv* probe was not significantly different from that seen for AKXL-21-derived RNA. RNA from AKXL-29 or C57L (*emv-11*$^-$,*13*$^-$,*14*$^-$) mice was used as a negative control in these experiments. In other experiments, Northern (RNA blot) analysis showed that the RNA transcripts were the expected size of the genome size 8.2-kb transcript (data not shown). The spliced *env* transcript was not detected in RNA samples from either *emv-14*$^+$ AKXL-5 tissue or from *emv-11*$^+$ *emv-13*$^+$ AKXL-9 positive control tissue, perhaps reflecting a low steady-state level of spliced *emv* message in general. The data are consistent with the notion that *emv-14* is transcribed at a level similar to that seen for *emv-11*.

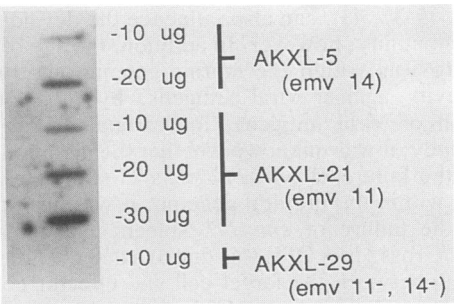

FIG. 1. AKXL liver RNA blot hybridization with *emv* probe. Total RNA (10, 20, or 30 μg, as indicated) was blotted onto a nitrocellulose membrane and hybridized with the *emv* probe as detailed in Materials and Methods. The hybridized blot was washed under standard high-stringency conditions and exposed for 4 days to X-ray film as described in Materials and Methods. The *emv* genotypes of the strains from which the RNA was isolated are as follows: AKXL-5, *emv-14*; AKXL-21, *emv-11*; AKXL-29, *emv-11*$^-$ and *emv-14*$^-$. AKXL-29 served as a negative control for virus expression.

Isolation and quantitation of EMV from AKXL mice. To further explore the expression of ecotropic virus in *emv-14*-positive mice relative to *emv-11*-positive mice, the relative titers of these viruses from the relevant strains of AKXL mice were quantitated by the XC plaque assay. Virus isolates were obtained from AKXL strains with the following *emv* genotypes: AKXL-21 (*emv-11*$^+$), AKXL-9 (*emv-11*$^+$ *emv-13*$^+$), AKXL-16 (*emv-11*$^+$ *emv-13*$^+$), AKXL-28 (*emv-13*$^+$), and AKXL-5 (*emv-14*$^+$). These virus isolates were quantitated by the XC plaque assay to determine the relative levels of viremia in the different AKXL strains. Figure 2 shows viral titers from a representative experiment. The viral titer from the *emv-14*$^+$ AKXL-5 strain was consistently found to be within the range of titers observed for the *emv-11*$^+$ strains, confirming that *emv-14* is antigen expression competent as well as message expression competent. The *emv-13*$^+$ AKXL-28 strain was negative for infectious ecotropic virus, as expected, since *emv-13* is known to be replication defective (9). There is the potential for increased viral titers in *emv-11*$^+$ *emv-13*$^+$ mice because the defective *emv-13* may be pseudotyped or complemented by *emv-11* (as is known to occur for *emv-3* with *emv-13* [9]). Such complementation may not explain the consistently observed increased virus titers and RNA expression of AKXL-9 mice, however, since AKXL-16 mice are also *emv-11*$^+$ *emv-13*$^+$

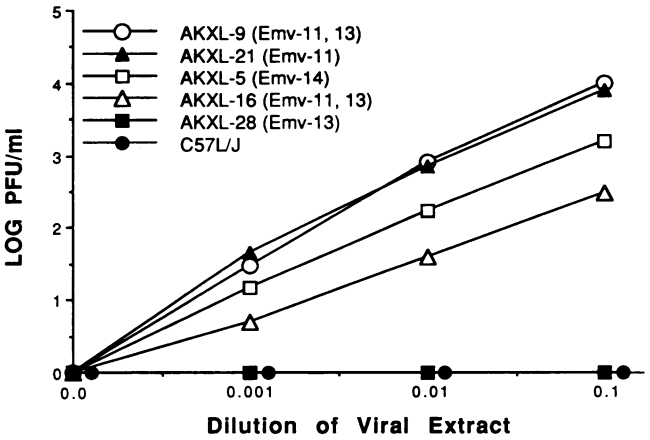

FIG. 2. Viral titers of AKXL mice. The viral titer of the AKXL-5 strain is compared with that of other AKXL strains by the XC plaque assay as given in Materials and Methods. The AKXL *emv* genotypes are as indicated.

but show somewhat decreased titers in comparison. Extracts from the C57L parental strain were also found to be negative, as expected. C57L mice have been reported to be *emv-16*$^+$ *emv-17*$^+$ (6) but were not viremic when assayed by the XC plaque assay (Fig. 2), indicating that these loci do not encode replication- and expression-competent *emv*. In fact, Buchberg et al. (6) maintain that the "*emv-17* allele" found in C57L mice is not even associated with an ecotropic provirus, but is defined on the basis of a restriction fragment length polymorphism associated with the viral preintegration site.

Preparation and analysis of SC.K^b cells. We wished to determine whether sequences within the *emv-14* virus were responsible for the failure of spleen cells from *emv-14*$^+$ AKXL-5 mice to stimulate anti-AKR/Gross virus CTL. Our approach was to use virus isolates from AKXL-5 mice, and from AKXL-21 mice (*emv-11*$^+$) as a positive control, to infect cells in culture and then determine whether these infected cells function as target cells for *H-2*^{b} restricted anti-AKR/Gross virus CTL. One would predict that *emv-14*$^+$ *H-2*^{b} targets would fail to be lysed if the defect responsible for the failure of AKXL-5 spleen cells to stimulate CTL were within the *emv-14* provirus and at the level of recognition between effector and viral antigen-positive cell.

In order to infect virus isolated from AKXL mice of various *emv* genotypes into cells in culture, it is important that the recipient cells be virus negative. The use of cells, such as L cells, that are already positive for murine leukemia viruses can result in significant background levels of nonspecific lysis in the antiviral CTL assay (2). We chose the SC-1 continuous line of wild mouse embryo cells because this line is negative for ecotropic viruses by several criteria. When SC-1 cells are used in the XC plaque assay for ecotropic viruses, no plaques are formed. A broad-spectrum goat anti-Rauscher gp70 antiserum fails to stain these cells significantly above background levels in flow cytometric analysis (data not shown). Culture of SC-1 cells in the presence of iododeoxyuridine (IUdR), an iodinated pyrimidine analog known to activate latent EMV proviruses with high frequency (1, 54), failed to induce viral expression as assayed by the XC plaque assay or by flow cytometric analysis (data not shown). Southern blot analysis with genomic DNA from SC-1 cells digested with the restriction endonuclease *Bam*HI failed to show any hybridization signal with the *emv* probe (data not shown). *Bam*HI digests EMV proviruses internally at conserved sites on both sides of the 334-bp *Sma*I probe sequence, giving a characteristic 3.3-kb fragment (29). The SC-1 cell line therefore appeared to be a good virus-negative recipient for infection with virus isolates from various AKXL *emv* genotype strains.

It was also necessary that the infected SC-1 cells be histocompatible with the anti-AKR/Gross virus CTL generated from C57BL/6 responder mice. Previously it was shown, by examining the ability of various antibodies to block cell-mediated lysis, that the K antigen rather than the D antigen restricted these anti-AKR/Gross virus CTL (23). Since SC-1 cells are derived from wild mouse embryo cells, their *H-2* haplotype was unknown to us. It was confirmed that the SC-1 *H-2* locus was not of the *b* haplotype. There was no significant staining with either anti-*K*^{b} (HB41) or anti-*D*^{b} (MCA169 or HB19) monoclonal antibodies above background levels of staining as assayed by flow cytometry with indirect immunofluorescence (Fig. 3). In addition, the monotypic anti-*H-2* class I monoclonal antibody M1/42, which binds efficiently to *H-2K*^{b} and most other class I antigens, did not bind significantly to SC-1 cells (data not shown), indicating either that SC-1 cells have low levels of *H-2* antigens or that there is poor cross-reactivity with the endogenous *H-2* antigens found in SC-1 cells. SC-1 cells were therefore made to be *H-2K*^{b} positive by transfection (see Materials and Methods) with the *K*^{b} gene maintained in the plasmid pTCF along with the Neo^r gene, conveying resistance to the drug G418 (3). The bulk population of cells that survived G418 drug selection was sorted for those SC.K^b cells expressing the highest concentrations of K^b antigen on the cell surface as determined by flow cytometry analysis with either the anti-*H-2K*^{b} monoclonal antibody HB41 or the monotypic anti-*H-2* monoclonal antibody M1/42 and indirect immunofluorescence (Materials and Methods). This strategy was used to select a population of cells with the highest levels of expression of cell surface K^b antigen for use as targets for the *H-2*^{b}-restricted anti-AKR/Gross virus CTL. The resultant SC.K^b population of cells had an increase in *K*^{b} antigen expression that was significantly above the value observed for untransfected SC-1 cells and close to the levels of expression seen for the positive control SL1 (*H-2*^{b}) tumor cells (Fig. 3). The population of SC.K^b cells was reanalyzed after a period of weeks, confirming that *K*^{b} antigen expression was stable. SL8 (*H-2*^{k}) tumor cells were negative for binding the anti-*D*^{b} and the anti-*K*^{b} monoclonal antibodies, as expected.

As previously mentioned, Green et al. (23) used monoclonal antibodies specific for the *K*^{b} antigen or for the *D*^{b} antigen to determine that only the anti-*K*^{b} antibody could prevent lysis by anti-AKR/Gross virus CTL of the appropriate target cells. It was not known whether this was because the *D*^{b} antigen simply could not act as a restricting element in this system under any circumstances or whether it did not act as a restricting element because of the presence of the dominant *K*^{b} antigen. We decided to analyze the class I restriction elements for the anti-AKR/Gross virus CTL further by transfecting SC-1 cells with a plasmid containing the *D*^{b} gene as described in Materials and Methods. Expression of *D*^{b} antigen on SC.D^b cells approached the *D*^{b} expression seen on SL1 positive control cells, suggesting that the SC.D^b cells had sufficient levels of expression for recognition by antiviral CTL (Fig. 3). Incidentally, the monotypic M1/42 monoclonal antibody, with broad specificity for *H-2* class I antigens, did not react with SC.D^b cells,

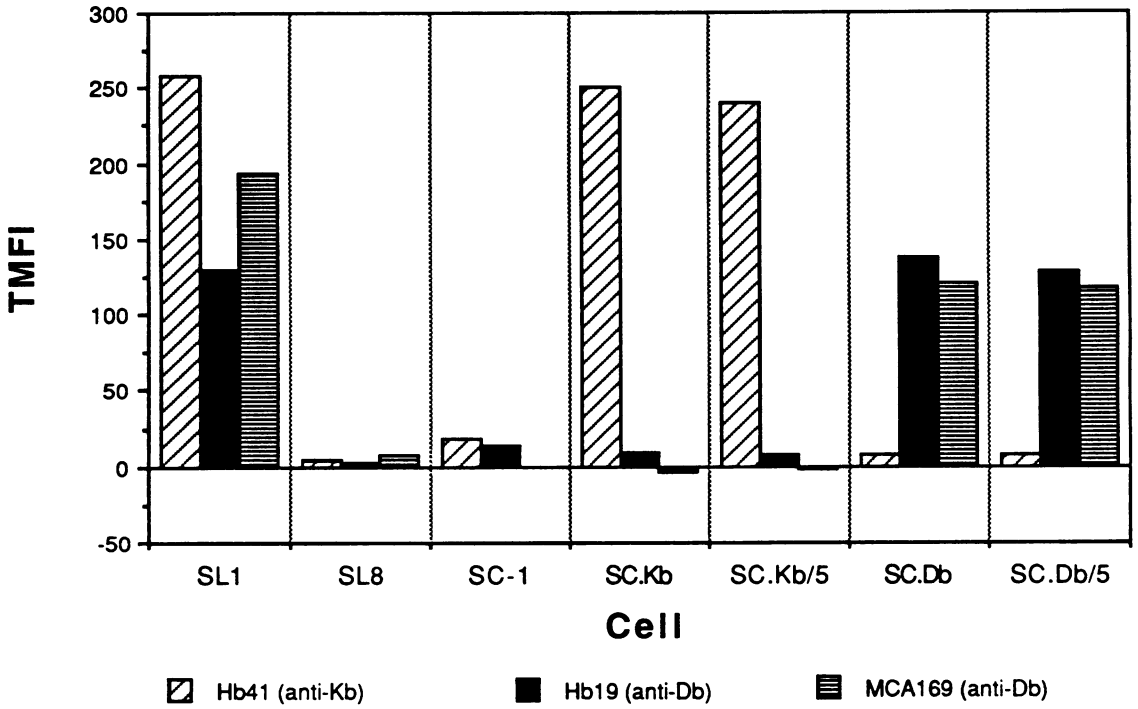

FIG. 3. SC-1 cells were transfected with the K^b gene or the D^b gene as detailed in Materials and Methods. The transfected cells were sorted and analyzed after sorting by flow cytometry. The antibodies used for the postsort analyses were HB41, specific for $H\text{-}2K^b$, and HB19 or MCA169, specific for $H\text{-}2D^b$. TMFI, Total mean fluorescence intensity (arbitrary units).

a finding that is consistent with the specificity for this antibody published previously (57, 58).

Allogeneic CTL functional analysis of SC.K^b and SC.D^b cells. To determine whether the expression of K^b antigen on the surface of the SC.K^b sorted cell population was sufficient for efficient lysis by CTL, these cells were labeled with ^{51}Cr for use as targets against allogeneic anti-$H\text{-}2^b$ CTL generated by stimulating spleen cells from DBA/2J ($H\text{-}2^d$) responder mice with irradiated spleen cells from C57BL/6J ($H\text{-}2^b$) mice (Materials and Methods). Representative data are shown in Fig. 4. SC.K^b cells that had been sorted for optimal K^b antigen expression were lysed substantially by the anti-$H\text{-}2^b$ allogeneic CTL. The sorted SC.D^b target cells were lysed by the allogeneic CTL with an efficiency approaching that seen for the SC.K^b cells. The use of untransfected SC-1 cells as targets resulted in background levels of lysis, as expected. The positive control SL1 tumor target cells of $H\text{-}2^b$ haplotype were efficiently lysed by the anti-$H\text{-}2^b$ CTL. YAC ($H\text{-}2^a$) cells were not lysed, indicating that cell lysis was due to allogeneic CTL rather than to NK cells, and showing that these CTL were specific for $H\text{-}2^b$. These findings provide evidence that the SC.K^b and the SC.D^b cell populations are efficient targets for allogeneic CTL and therefore should have sufficient K^b and D^b antigen expression, respectively, to be lysed by antiviral CTL after infection with virus isolates from various AKXL mice.

Infection of SC.K^b and SC.D^b cells with EMV isolates from AKXL mice. In order to analyze the ability of *emv-11* versus *emv-14* viral antigens to serve as targets for anti-AKR/Gross virus CTL, the SC.K^b transfected cells were infected with virus isolates (described above) from AKXL mice having the various *emv* genotypes. After the infection was allowed to spread laterally by extended cell culture, virus titers in the EMV-infected SC.K^b lines were determined by the XC plaque assay. *emv-11*-containing SC.K^b/9 or SC.K^b/21 cells and *emv-14*-containing SC.K^b/5 cells had equivalently high viral titers (Fig. 5). The virus titers of these cells were reconfirmed after a period of time to show that virus production was stable. The data confirm that SC.K^b cells were infected by virus isolated from the various AKXL mice. Similar virus titers were shown for EMV-infected SC.D^b cells (data not shown).

Antiviral CTL lysis of EMV-infected SC.K^b and SC.D^b cells. SC.K^b/5 cells were used as targets for $H\text{-}2^b$-restricted anti-AKR/Gross virus CTL lysis to determine whether *emv-14*, upon infection into SC.K^b cells, continued to escape recognition by antiviral CTL. Representative data are shown in Fig. 6A and B. SC.K^b/5 cells were not lysed by such CTL significantly above the control values (obtained by using effector cells not restimulated by antigen in vitro, i.e., unstimulated effector cells). In sharp contrast, SC.K^b/9 and SC.K^b/21 target cells, both containing *emv-11*, were efficiently recognized and lysed by antiviral CTL. These *emv-11*$^+$ target cells were lysed to a similar extent as SC.K^b cells infected with AKR 623 virus, a cloned provirus derived from an SC-1.Akv-1 congenic cell line (38). The two positive control ($H\text{-}2^b$, GCSA$^+$) tumor cell targets SL1 and E♂G2 were lysed efficiently, and the variant cl.18.5 was lysed poorly, as is usually seen for bulk anti-AKR/Gross virus CTL specifically directed against these tumor cell targets (16). The failure of effector cells to lyse YAC targets confirmed that NK cells were not present. As expected, relatively low levels of lysis of SC-1 target cells were observed because they were neither $H\text{-}2^b$ nor *emv*$^+$; SC.K^b cells were poorly lysed because they were not *emv*$^+$; and SC-1/21 cells were poorly lysed because they were not $H\text{-}2^b$. These data show that the defect responsible for the previously observed failure of AKXL-5 spleen cells to stimulate

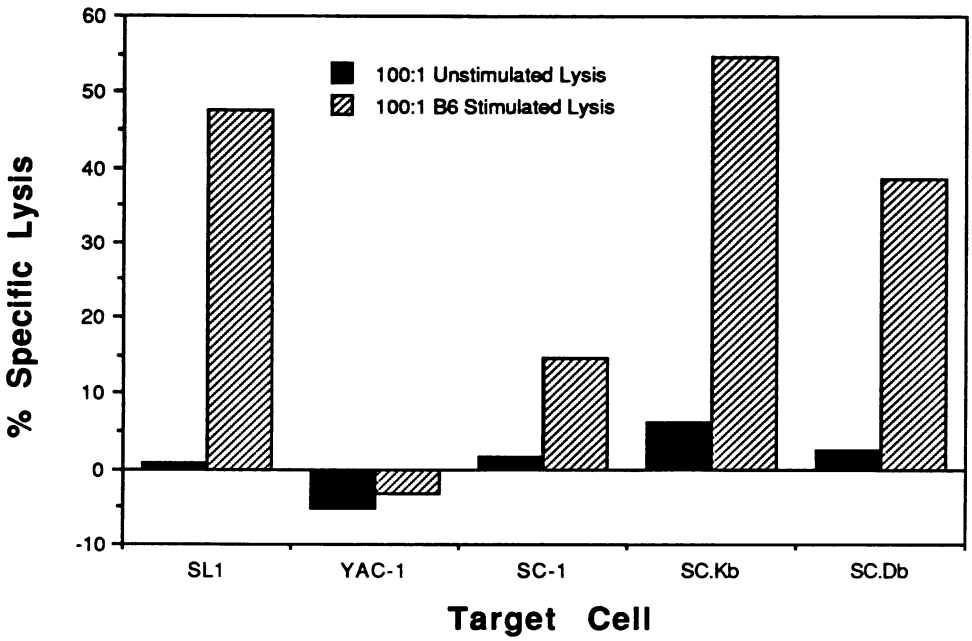

FIG. 4. Allogeneic CTL lysis of SC.K^b and SC.D^b cells. SC.K^b cells and SC.D^b cells were transfected with the K^b gene or the D^b gene, respectively, as given in Materials and Methods. Cells positive for class I K^b or D^b antigen expression were G418 selected, sorted, and analyzed with monoclonal antibodies and by flow cytometry as indicated in the legend to Fig. 3. The resultant SC.K^b and SC.D^b cells were then analyzed for their ability to function as target cells in a ^{51}Cr release assay against allogeneic anti-H-2^b CTL. Allogeneic anti-H-2^b CTL were generated by stimulating spleen cells from DBA/2J (H-2^d) responder mice with irradiated spleen cells from C57BL/6J (H-2^b) mice as detailed in Materials and Methods. Unstimulated effector cells were used as negative controls. SL1 are H-2^b Gross virus-positive tumor cells, and YAC is a highly NK-sensitive target cell. The data for an effector-to-target cell (E:T) ratio of 100:1 are shown. Ratios of 20:1 and 4:1 were also used, and the percent lysis (data not shown) was titrated as expected.

anti-AKR/Gross virus CTL is at the level of recognition between virus-infected cell and effector CTL or pre-CTL and that the mutation responsible for this defect is most likely located within the *emv-14* provirus.

Neither *emv-11*- nor *emv-14*-infected SC.D^b cells were efficiently lysed, as shown by representative data in Fig. 6C.

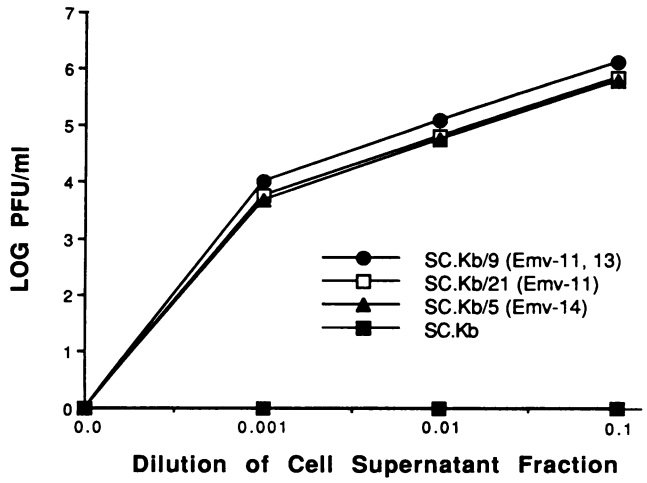

FIG. 5. Viral titers of infected SC.K^b cells. SC.K^b cells were infected with virus isolated from various AKXL strains. After a period of weeks, the infected SC.K^b cells were analyzed by the XC plaque assay as detailed in Materials and Methods to titer the virus expression. SC.K^b/9, SC.K^b/21, and SC.K^b/5 are SC.K^b cells infected with virus isolated from AKXL-9, AKXL-21, and AKXL-5 mice, respectively.

This failure occurred despite the fact that SC.D^b cells are capable of being lysed by anti-H-2^b allogeneic CTL (Fig. 4). In the same experiment, SC.K^b/9, SC.K^b/21, and SC.K^b/623 targets were killed at levels similar to those found in other experiments (data not shown), indicating that these particular bulk CTL were effective as usual against *emv-11*-associated fibroblast targets. The results indicate that anti-AKR/Gross virus CTL are unable to recognize these viral antigens in the context of the D^b class I antigen even when the dominant K^b class I antigen is absent. This finding is in contrast to work by others, who maintain that *env* antigens recognized by anti-Gross virus CTL, raised by a somewhat different sensitization protocol, are restricted significantly by D^b in addition to K^b (51).

It was important to confirm that the failure of anti-AKR/Gross virus CTL to lyse SC.K^b/5 cells was not due to downregulation of the expression of the transfected class I gene by *emv-14* and not by *emv-11*. Others have shown that MHC class I antigen expression can be regulated by virus infection (reference 12, for example). When SC.K^b or SC.D^b cells were infected with virus isolated from *emv-14*$^+$ mice, the expression of K^b or D^b antigen, respectively, was not decreased (Fig. 3). In addition, SC.K^b/5 cells were lysed by anti-H-2^b allogeneic CTL (DBA anti-C57BL/6, generated as for Fig. 4) as efficiently as SC.K^b/9 cells. At an effector-to-target cell ratio of 100:1, for example, the lysis of SC.K^b/5 and SC.K^b/9 target cells was 62 and 61%, respectively, relative to 65% lysis of the positive control H-2^b EδG2 tumor cells (data not shown). The lack of killing of YAC-1 (H-2^a) target cells (4.5% lysis) in this particular experiment indicated that the lysis was H-2^b specific and due to allogeneic CTL rather than to NK cells. A decrease in MHC class

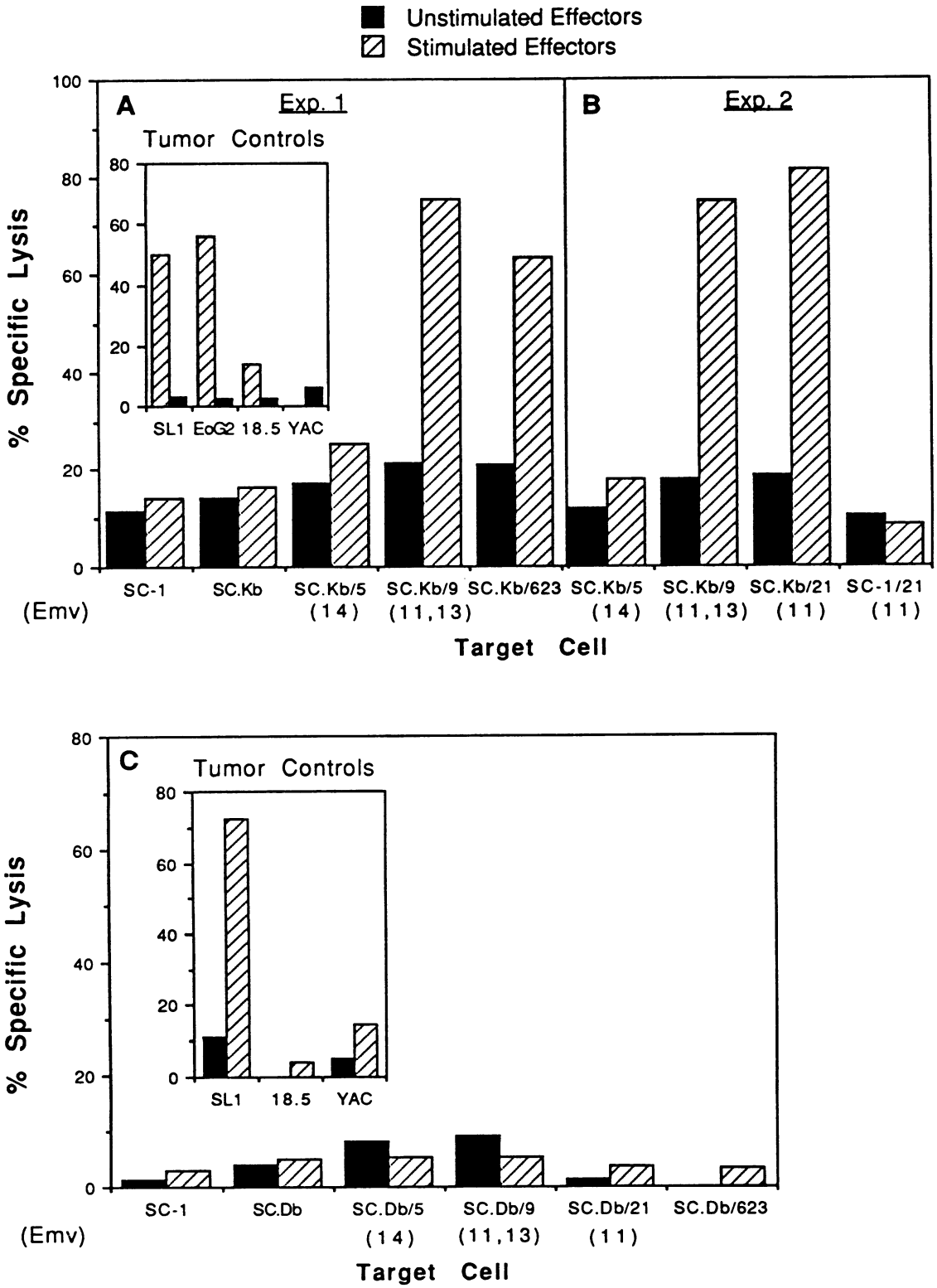

FIG. 6. Antiviral CTL lysis of EMV-infected SC.K^b and SC.D^b cells. SC.K^b and SC.D^b cells infected with virus isolates from various AKXL strains were analyzed for their ability to be lysed in a ^{51}Cr release assay by anti-AKR/Gross virus CTL. Antiviral CTL were generated by stimulating B.GV-primed C57CL/6J spleen cells in vitro with irradiated SL1 cells as detailed in Materials and Methods. The *emv* genotypes of the infected SC.K^b and SC.D^b cells are indicated along the abscissa. SC.K^b/623 and SC.D^b/623 cells are SC.K^b or SC.D^b cells, respectively, infected with AKR 623 virus. SL1 and EδG2 cells are *H-2*^{b} Gross virus-positive control tumor cells. Cl.18-5 serves as a negative control for anti-AKR/Gross virus CTL. YAC is a highly NK-sensitive target cell. The effector-to-target cell (E:T) ratios of the data shown were 100:1. Ratios of 20:1 and 4:1 were also used, and the percent lysis (data not shown) was titrated as expected.

TABLE 1. Viral epitope expression of infected SC.K^b cells[a]

Cells	Antibody	% Positive[b]	TMFI[c]
gp70 analysis			
SC.K^b	Anti-gp70^a	7	62
	Anti-gp70^b	3	33
	Anti-gp70^c	2	27
	Anti-gp70^d	3	23
	Anti-gp70^f	0	3
	Anti-gp70^g	0	4
	Anti-gp70^h	0	5
SC.K^b/5 (*emv-14*)	Anti-gp70^a	90	542
	Anti-gp70^b	79	383
	Anti-gp70^c	4	54
	Anti-gp70^d	51	209
	Anti-gp70^f	80	375
	Anti-gp70^g	16	77
	Anti-gp70^h	1	15
SC.K^b/9 (*emv-11*, *emv-13*)	Anti-gp70^a	93	551
	Anti-gp70^b	85	412
	Anti-gp70^c	1	26
	Anti-gp70^d	45	181
	Anti-gp70^f	85	389
	Anti-gp70^g	21	97
	Anti-gp70^h	2	20
SC.K^b/21 (*emv-11*)	Anti-gp70^a	91	542
	Anti-gp70^b	85	403
	Anti-gp70^c	2	28
	Anti-gp70^d	43	180
	Anti-gp70^f	84	382
	Anti-gp70^g	20	97
	Anti-gp70^h	2	20
gag analysis			
SC.K^b	Anti-glycosylated *gag*	−3	−5
	Anti-p12	−3	−9
SC.K^b/5 (*emv-14*)	Anti-glycosylated *gag*	70	165
	Anti-p12	71	183
SC.K^b/9 (*emv-11*, *emv-13*)	Anti-glycosylated *gag*	70	150
	Anti-p12	72	164
SC.K^b/21 (*emv-11*)	Anti-glycosylated *gag*	80	173
	Anti-p12	82	192

[a] SC.K^b cells prepared as described in the legend to Fig. 4 and infected with the various virus isolates prepared as described in Materials and Methods were analyzed for their expression of gp70 antigen (representative data shown) or for their expression of *gag* antigen (representative data shown) by flow cytometry. The monoclonal antibodies are directed against the different viral epitopes as described in Materials and Methods.

[b] Values were corrected by subtracting the values for the isotype controls (Materials and Methods).

[c] TMFI = total mean fluorescence (arbitrary units).

I antigen expression due to *emv-14* infection cannot therefore account for any defect seen in the ability of SC.K^b/5 cells to be lysed by CTL.

Expression of virus antigens on virus-infected SC.K^b cells. To further characterize viral antigen expression in the EMV-infected SC.K^b cells, the cells were analyzed by flow cytometry with a panel of antiviral antibodies. Cytofluorographic data with monoclonal antibodies specific for seven different epitopes on the envelope antigen gp70 and two *gag* determinants are shown in Table 1. The results confirm that the cells were infected and expressing significant levels of viral antigen. The data were also used to look for differences in expression between *emv-11* and *emv-14* that might correlate with the failure of *emv-14*-infected cells to be recognized by anti-AKR/Gross virus CTL. Surprisingly, there were no significant differences in the pattern of expression for the various epitopes analyzed when comparing SC.K^b/9 (*emv-11*, *emv-13*) or SC.K^b/21 (*emv-11*) cells with SC.K^b/5 (*emv-14*) cells by this approach (Table 1). Interestingly, the gp70^c epitope was not expressed to a significant extent on any of the EMV-infected cells, despite the ability of the anti-gp70^c monoclonal antibody to react strongly with positive-control SL1 tumor cells (data not shown). In other cytofluorographic experiments, the expression of three monoclonal antibody-defined epitopes on the p15(E) envelope antigen and the use of four additional *gag*-specific antibodies demonstrated no significant differences in expression between *emv-11*- and *emv-14*-infected cells (data not shown). The data suggest that if there is a mutation within the coding sequence of an *emv-14 gag*- or *env*-derived viral antigen that correlates with CTL dysfunction, it may be a point mutation, for example, that is not detectable with these particular antibodies.

DISCUSSION

It was previously shown that spleen cells from *emv-14*$^+$ AKXL-5 mice fail to stimulate an anti-AKR/Gross virus CTL response in a mixed lymphocyte culture with primed C57BL/6 responder spleen cells, whereas *emv-11*$^+$ AKXL strains do stimulate a CTL response (21). One possible explanation for this failure is that there is a mutation within a coding sequence portion of the viral genome at a site critical for CTL recognition. Such a mutation could allow *emv-14*-positive tumor cells to avoid recognition and lysis by antiviral CTL. This would represent one mechanism by which tumors may escape cellular immunity and become established. It was therefore of interest to analyze the mechanism by which *emv-14*$^+$ cells fail to be recognized by anti-AKR/Gross virus CTL. It was important to first determine whether the mutation responsible for the failure of AKXL-5 spleen cells to stimulate a response was within the *emv-14* proviral genome or whether the mutation was at some other locus within the murine genome. It was also important to determine whether the relevant mutation was at the level of recognition between target cell and effector cell. It is shown here that when virus isolated from the *emv-14*$^+$ AKXL-5 strain is used to infect SC.K^b cells, the resultant SC.K^b/5 cells are productively infected (Fig. 5 and Table 1) but continue to evade recognition and lysis by *H-2^b*-restricted anti-AKR/Gross virus CTL (Fig. 6). Yet in a parallel positive-control experiment, *emv-11*$^+$ SC.K^b/21 target cells were lysed by these antiviral CTL, indicating that there is nothing inherent about EMV-infected SC.K^b cells that would prevent them from being recognized and lysed efficiently by anti-AKR/Gross virus CTL. Overall, the data indicate that the mutation responsible for CTL dysfunction is at the level of recognition between target cell and effector cell and is almost certainly within the *emv-14* proviral genome, since the resistance of *emv-14*$^+$ cells to antiviral CTL was maintained upon isolation of virus from AKXL-5 cells and infection into SC.K^b cells. The fact that the *emv-14* provirus has reintegrated into different loci within the murine genome in SC.K^b/5 cells relative to AKXL-5 cells (Southern data not shown) is consistent with the mutation's being within the *emv-14* provirus and unaffected by flanking murine sequences.

The facts that AKXL-5 mice are viremic and that virus message and cell surface antigens are expressed at levels similar to those for *emv-11* (Fig. 1 and 2, Table 1) indicate that *emv-14* is expression competent and has a functional life cycle. The relative titers for AKXL-5 and AKXL-16 mice, as shown in Fig. 2, are especially important, since anti-AKR/Gross virus CTL were stimulated by *emv-11*-containing

AKXL-16 spleen cells. These data, along with the RNA and antigen quantitations, support the hypothesis that *emv-14* virus expression is not quantitatively limiting the CTL recognition process.

Green and Rich (unpublished) have shown that when AKXL-5 (*emv-14*$^+$) spleen cells are first treated with IUdR, in the presence of lipopolysaccharide to stimulate incorporation of IUdR into DNA, and then used to stimulate an antiviral CTL response by primed C57BL/6 spleen cells, anti-AKR/Gross virus CTL still are not stimulated. This finding, along with the data presented here showing that *emv-14* is expression competent, is consistent with the relevant mutation's being within the coding sequence of an expressed viral antigen critical for CTL recognition and not at a regulatory site that may act to repress expression.

It would seem logical that an envelope antigen, especially gp70, might be the target for CTL recognition and function since this antigen is displayed on the cell surface and since envelope antigens are recognized by and stimulate the proliferation of antiviral CTL in other retroviral systems (for example, see references 11 and 67). Alternatively, other retroviral antigens may be targets for the CTL antigen receptor. It has even been shown that CTL specific for the human immunodeficiency virus (HIV) *pol* gene product, reverse transcriptase, were found in blood samples from HIV-1-seropositive individuals (68). This finding occurs despite the fact that reverse transcriptase is normally thought to reside within the virion in association with the RNA genome, and may be due to viral antigen processing and presentation in association with MHC antigen. In fact, there are now numerous examples of viral antigens that are localized intracellularly and yet are recognized by virus-specific CTL. Specific examples include the influenza virus nucleoprotein (65, 66) and viral polymerases (4), simian virus 40 T antigen (44, 63), murine cytomegalovirus immediate-early regulatory protein (34), human cytomegalovirus 72-kilodalton immediate-early protein (5), lymphochoriomeningitis virus nucleoprotein (25), and herpes simplex virus immediate-early proteins (41). In some cases it has been confirmed that these virus antigens are serologically undetectable on the cell surface (for example, see reference 41). In some cases virus antigens are known to be largely localized in the nucleus but are detectable at the cell surface as determined by the inhibition of effector cell activity by virus antigen-specific antibody (for example, see references 7 and 48). These findings are compatible with the hypothesis that a processed peptide fragment bound to its MHC restriction element is on the cell surface and is recognized by antiviral CTL, but may or may not be recognizable by virus-specific antibodies. This is understandable in light of analyses by Grey and by Berzofsky of peptides bound to MHC molecules having a molecular weight range of 2,000 to 10,000 (discussed in reference 42). These data indicate that peptides can range from 10 to 20 amino acids in size to a much larger size at which parts of the peptide are "spilling over" the side of the MHC molecule. The presence of these larger MHC-bound viral peptides may account for the ability of anti-simian virus 40 T antigen monoclonal antibody (48) or anti-human cytomegalovirus nuclear inclusion body protein antibody (7) to block the lysis of virus-positive or virus-coated target cells, respectively, by anti-virus-specific CTL. This is an alternative hypothesis to the suggestion that cell degradation due to viral pathogenesis may account for the access of antiviral CTL to internal antigens.

There is evidence, however, that it is the gp70 envelope antigen that is the major target for anti-AKR/Gross virus CTL. Altered expression of the gp70 envelope antigen of two variant subclones, cl.18-5 and cl.18-12, derived from a CTL-susceptible tumor cell line of AKR.H-2^b origin, correlate with the failure of these cells to be lysed efficiently by anti-AKR/Gross virus bulk CTL. Treatment of these variant subclones with IUdR to induce the expression of endogenous latent EMV restores the expression of gp70 epitopes and the susceptibility of these cells to lysis by anti-AKR/Gross virus CTL (40). In addition, when *env*-defective cl.18-5 is used to stimulate anti-AKR/Gross virus CTL, the lysis of tumor or fibroblast target cells that are otherwise lysed efficiently by SL1- or E♂G2-stimulated CTL drops dramatically (data not shown). These findings suggest that the majority of the bulk CTL used here are *env* specific. These data are in contrast to data by others, who maintain that anti-AKR/Gross virus CTL recognize both *gag* and *env* gene products with comparable intensities (51, 52). It was therefore of great interest to determine whether the *gag* or especially the *env* antigen expression profile for *emv-14*$^+$ SC.K^b/5 cells was different from that for *emv-11*$^+$ SC.K^b/21 cells. Table 1 shows that there are no significant differences in antigen profile as analyzed by antiviral monoclonal antibodies and flow cytometry. These results were intriguing since the cl.18-5 and cl.18-12 variant subclones mentioned above showed large differences in the expression of gp70 epitopes relative to the AKR.H-2^b SL1 parental line. The data indicate that if gp70, or one of the other viral antigens analyzed by flow cytometry, is the target for CTL recognition against *emv-14*$^+$ cells, then there must be a mutation, perhaps a point mutation or a few point mutations or a small insertion or deletion, that is responsible for the loss of CTL function but is undetectable with the antibody reagents available for analysis.

It is commonly thought that viral antigens provide multiple determinants for CTL stimulation. How then could a small mutation that is not detectable by antibody analysis be responsible for abrogating a CTL response? There is precedence in the literature for the recognition of a limited number of immunodominant epitopes by antiviral CTL. For example, a major population of murine influenza virus A-specific CTL recognize the viral nucleoprotein (64); these investigators then determined via deletion mutant analysis of the NP gene that D^b-restricted polyclonal CTL are predominantly directed at a 59-amino-acid region of NP. The majority of CTL against lymphocytic choriomeningitis virus glycoprotein (GP) recognize targets expressing only a 22-amino-acid region (30, 45). For the HIV gp160^{env} glycoprotein, the use of overlapping peptides in a vaccinia virus expression system revealed that a single 15-residue site was immunodominant for CTL recognition in responder *H-2*^{d} mice (61). In fact, the same group recently synthesized two 15-mer peptides derived from this immunodominant region and based on two different HIV-1 isolates. The peptides differed in 6 of 15 residues, but the reciprocal exchange of only one of the hypervariable residues was sufficient to interchange the specificities of isolate-specific CTL (62). It is also possible that a point mutation, or small insertion or deletion, within a coding region of the virus may cause a conformational change which destroys more than one determinant involved in CTL recognition. A mutation within overlapping epitopes would also affect more than one determinant. Finally, because the EMVs are carried endogenously as proviruses in the murine germ line, there may be fewer EMV determinants recognized by CTL because of exposure of thymic cells to similar C57BL/6 proviral determinants. That is, selective

pressures like those acting to induce tolerance may be occurring.

Thus, in the C57BL/6 system, it is possible that a small coding sequence mutation in the *emv-14* virus is responsible for the failure of *emv-14*$^+$ SC.K^b/5 cells to be lysed by anti-AKR/Gross virus CTL. In light of the possibility that retroviruses such as HIV may use hypervariable *env* regions to escape cellular immunity (see references 61 and 62, for example), it will be interesting to further define the nature of this mutation. It is less clear whether these findings will hold in other strains, especially high leukemic strains such as AKR/J. Although this paper deals with N-ecotropic proviruses from AKR/J mice, we and others have noticed a number of differences in the ability of strains of the *H-2*^{b} and *H-2*^{k} haplotypes to mount a response against AKR-derived leukemic viruses and the tumors they induce. Both AKR/J (*H-2*^{k}) and AKR.H-2^b mice are nonresponders to N-ecotropic proviral determinants because their immune system has seen the virus starting from before birth (e.g., reference 37). The functional immunogenicity of EMVs would therefore be difficult to study within the AKR/J system. In addition, *H-2*^{k} haplotype mice (most notably C57BL/6.H-2^k [19]) are poor responders in general for eliciting anti-AKR/Gross virus CTL. Others have shown that the majority of leukemias from AKR mice are poorly immunogenic in the AKR strain (56). In the same study, one unusual leukemia, 369, was highly immunogenic and induced CTL which killed 369 cells in vitro; in contrast, most of the AKR effector cells derived by immunizing and restimulating in vitro with the various immunizing leukemias failed to lyse the immunizing tumor cells. In addition to a poor anti-AKR/Gross virus CTL response on the part of AKR mice, EMVs have high mutation rates. These factors together are likely to contribute significantly to the exceptionally high incidence of leukemia in AKR mice.

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